Environmental Toxicology and Chemistry—Volume 00, Number 00—pp. 1–11, 2021
Received: 7 December 2020 | Revised: 7 January 2021 | Accepted: 2 March 2021

Environmental Toxicology

Impacts to Larval Fathead Minnows Vary between Preconsumer and Environmental Microplastics

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Abstract: Microplastics are a complex suite of contaminants varying in size, shape, polymer, and associated chemicals and are sometimes referred to as a "multiple stressor." Still, the majority of studies testing hypotheses about their effects use commercially bought microplastics of a uniform size, shape, and type. We investigated the effects of polyethylene and polypropylene microplastics purchased as preproduction pellets (referred to as "preconsumer") and a mixture of polyethylene and polypropylene collected from the environment (environmental microplastic). Embryo-stage fathead minnows were exposed to either the physical plastic particles and their leachates or the chemical leachates alone at an environmentally relevant (280 particles/L) or high (2800 particles/L) concentration for 14 d. The effects of microplastics differed by polymer type and presence of environmental contaminants, and effects can be driven by the physical particles and/or the chemical leachates alone. Larvae exposed to preconsumer polyethylene experienced a decrease in survival, length, and weight, whereas preconsumer polypropylene caused an increase in weight. Environmental microplastics caused a more drastic increase in length and weight and almost 6 times more deformities as the preconsumer microplastics. Although preconsumer microplastics caused effects only when organisms were exposed to both the particles and the chemical leachates, the environmental microplastics caused effects when organisms were exposed to the chemical leachates alone, suggesting that the mechanism of effects are context-dependent. The present study provides further support for treating microplastics as a multiple stressor and suggests that testing for effects with pristine microplastics may underestimate the true effects of microplastics in the environment. *Environ Toxicol Chem* 2021;00:1–12. © 2021 SETAC

Keywords: Plastic pollution; Multiple stressor; Ecotoxicology; Endocrine disruption; Fathead minnows; *Pimephales promelas*

INTRODUCTION

The global ubiquity of microplastics (plastic <5 mm) in the environment has led to widespread investigation of their biological effects in marine, freshwater, and, less frequently, terrestrial settings (Foley et al. 2018; Bucci et al. 2020). Testing the effects of microplastics has primarily involved conducting single-species laboratory studies to test the effects of a uniform type, shape, and size of microplastics at concentrations that are unrealistic compared to what is found in the environment (Paul-Pont et al. 2018). Furthermore, the majority of laboratory experiments expose organisms to preproduction microplastics, occasionally referred to as "pristine" microplastics. Preproduction microplastics are readily available from scientific supply stores in a variety of size ranges and are often spherical in shape. However, these microplastics are not representative of microplastics found in the environment, because of their uniformity in shape and size and because they lack the complex chemical cocktail that is associated with microplastics in the environment (Teuten et al. 2009). This chemical cocktail consists of unreacted oligomers from the production of the plastic itself, chemicals added to the plastic product during manufacturing, and environmental contaminants (persistent organic pollutants [POPs] and heavy metals) that sorb to the plastic from the environment (Rochman 2015). Although investigating the effects of preproduction or preconsumer microplastics is valuable, the results of these studies do not reflect the true effects of microplastic pollution on wildlife.

Despite the increase in studies that test the effects of microplastics, there is still no consensus on whether or not microplastics are an important threat to wildlife. In fact, a recent review of the literature showed that exposure to microplastics resulted in a statistically significant outcome in only 47% of tested effects (Bucci et al. 2020). Effects that have been demonstrated in laboratory studies include increased mortality

This article contains online-only Supplemental Data.
* Address correspondence to k.bucci@mail.utoronto.ca
Published online 20 April 2021 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5036

(Au et al. 2015), reduced feeding (Cole et al. 2015), reduced growth (Au et al. 2015), decreased clutch sizes (Sussarellu et al. 2016), decreased hatching success (Cole et al. 2015), decreased larval size (Besseling et al. 2014), and decreased offspring survival (Lee et al. 2013). The vast majority of these studies, however, were conducted with low–trophic level organisms, mostly crustaceans and mollusks. In fact, few studies have tested effects at higher trophic levels, despite the prevalence of microplastics at all levels of the food web (Provencher et al. 2019) and their ability to transfer between trophic levels (da Costa Araújo et al. 2020). In fish, reported effects include signs of liver stress (Rochman et al. 2013); changes to gene expression (Rochman et al. 2014); increased degranulation of primary granules, neutrophil extracellular trap release, and oxidative burst (Greven et al. 2016); and decreased hunting behavior and metabolism (Mattsson et al. 2015). However, many studies testing similar endpoints have reported "no significant effect" (see Cole and Galloway 2015; Davarpanah and Guilhermino 2015; Mazurais et al. 2015). The inconsistencies between studies in terms of whether or not an effect is detected are likely the consequence of ignoring the complexity and context of microplastics as an environmental contaminant (Paul-Pont et al. 2018; Bucci et al. 2020).

Recently, more studies have begun teasing apart the effects of microplastics with regard to their shape, size, and associated chemicals. For instance, studies have shown that irregularly shaped particles are more harmful than spherical particles (Gray and Weinstein 2017), that smaller particles are more harmful than larger particles (Earn et al. 2020), and that the type of plastic also affects the type and severity of effect (Lagarde et al. 2016). In addition, studies have shown that the type and severity of effects are different or exacerbated when the organism is exposed to environmental microplastics with sorbed contaminants compared to preproduction, or "virgin," microplastics. For example, Japanese medaka exposed to clean polyethylene exhibited signs of liver stress (Rochman et al. 2013) and changes to gene expression (Rochman et al. 2014), whereas the same polyethylene fragments with sorbed environmental contaminants caused exacerbated effects such as tumor growth (Rochman et al. 2013) and abnormal germ cell proliferation (Rochman et al. 2014). However, these studies also reported no significant effects on mortality and cell necrosis (Rochman et al. 2013) and gonadal abnormalities (Rochman et al. 2014).

Although research concerning the effects of microplastic pollution is growing and more studies are starting to treat microplastics as a complex suite of contaminants, significant gaps remain. There is an abundance of evidence from field observations and laboratory experiments that aquatic organisms at all trophic levels interact with or have the potential to ingest microplastic particles. And yet, the majority of effects testing uses low–trophic level organisms such as crustaceans and mollusks (Bucci et al. 2020). Further, many studies are conducted over short time periods, some exposing organisms for less than 1 d (Greven et al. 2016). Finally, the microplastics used in many studies lack environmental relevancy, and the endpoints being targeted are at the organismal or suborganismal level and thus are difficult to relate to ecological relevancy (although they can be informative for ecosystem-level processes). The aim of the present study was to investigate the effects of preconsumer and environmental microplastics to the hatching success, survival, and development of the fathead minnow (*Pimephales promelas*), a model organism historically used in ecotoxicity testing and an important prey species in freshwater lakes and rivers in North America (Ankley and Villeneuve 2006). The objectives of the present study were 1) to investigate the effects of 2 commonly produced and polluted plastic types, polyethylene and polypropylene; 2) to investigate the effects of microplastics with and without sorbed contaminants; and 3) to inform the mechanism of exposure, whether effects are caused by the plastic particles or the chemical leachates.

METHODS

Preparation of microplastics

Black polyethylene and black polypropylene pellets were obtained from Craft Pellets and ASPX, respectively. In addition, pieces of black plastic were collected from the shoreline of Lake Ontario (environmental microplastics) in Humber Bay Park East, Toronto, Ontario, Canada (43°37′43.8″N 79°28′29.5″W). Lake Ontario is the terminal lake in the Laurentian Great Lakes chain, and its watersheds are highly urbanized and industrialized. The Humber River, which flows into the Humber Bay, is a major source of pollution to Lake Ontario (Corcoran et al. 2015). Each piece of environmental microplastic was confirmed to be polyethylene or polypropylene through Fourier-transform infrared spectroscopic analysis. Equal parts of polyethylene and polypropylene by weight were included in the final environmental microplastic mixture. The collected plastic was then lightly scrubbed and rinsed with deionized water to remove the biofilm and cut into pellet-sized pieces. The polyethylene and polypropylene pellets and the environmental microplastic pieces were each ground into fragments using a burmill coffee grinder (Cuisinart® Supreme Grind™ Automatic Burr Mill) and sieved with stainless steel sieves to reach a size range of 150 to 500 µm, which is within the size range of food for larval fathead minnows. The fragments were rinsed through the sieve with deionized water, dried in an oven at 60 °C overnight, and stored in an airtight container. This process is not expected to change the morphology of the particles, based on the findings of Munno et al. (2018), who did not see changes in shape or size of particles digested with KOH at 60 °C.

Experimental setup and culturing

Fathead minnow (*P. promelas*) eggs were obtained from breeding pairs from the in-house culture stock at the Ontario Ministry of the Environment, Conservation and Parks (MECP). Fish maintenance and sampling followed protocols approved by the Ontario MECP Laboratory Services Animal Care Committee (approval number ATU-004-19). For each experiment,

eggs (<24 h postfertilization) were gently rolled off clay spawning tiles into a Petri dish. The eggs from each breeding pair were mixed together to be sorted randomly into twenty-five 500-mL beakers with preaerated dechlorinated laboratory water. Once the eggs hatched (~3 d postfertilization), the fish were fed once per day with newly hatched (48 h) brine shrimp, ad libitum, evenly across all treatments. The beakers were held in water baths maintained at 24 ± 1 °C with a 16:8-h light:dark photoperiod, in accordance with the MECP culturing conditions (SOPATU004 revision 4). The water baths were covered with plastic sheeting to prevent ambient microplastics from settling into the experimental vessels throughout the exposure. Water quality (temperature, dissolved oxygen, conductivity, ammonia) was monitored daily, in one replicate per treatment.

Fathead minnow exposures

Three separate 14-d exposures were conducted, each with different plastic (polyethylene, polypropylene, environmental microplastic), to investigate the physical and chemical effects of microplastics on the development and survival of larval fathead minnows. The 14-d exposure used is similar to the 7-d larval growth and survival applied by Environment Canada (2011); however, we used a longer exposure time to increase environmental relevance. Each experiment included 2 concentrations (280 and 2800 particles/L), 2 exposure scenarios (physicochemical and chemical), and a control (i.e., 5 treatments: physchem-low, physchem-high, chem-low, chem-high, control). The dose was renewed daily to maintain a consistent concentration in both exposure scenarios over time. Each treatment had 5 replicates ($n = 5$) with 20 individuals per replicate. During each experiment, hatching success, survival, and morphological deformities were monitored daily; and the average weight and length of the fish were recorded after 14 d of exposure (11–12 d posthatch).

The concentrations used for each plastic type consisted of an environmentally relevant dose of 280 particles/L (Dubaish and Liebezeit 2013) and a high dose of 2800 particles/L. Because of the different densities of each plastic, the doses were measured as a particle count per unit of water to maintain the same concentration across the 3 experiments. The concentrations in mass per unit volume corresponded to 1.82 and 18.2 mg/L for polyethylene, 1.43 and 14.3 mg/L for polypropylene, and 2.58 and 25.8 mg/L for environmental microplastic. The mass concentrations for each plastic type were obtained by counting 100 particles under a microscope and weighing them. This was repeated 10 times for each plastic type, and the average was taken.

The exposure scenarios were designed such that the larvae were exposed to either the plastic particles and their leachates ("physicochemical" scenario) or to the leachates alone ("chemical" scenario). For the preconsumer plastic types, the leachate may consist of constituent chemicals from each polymer and any plastic additives from manufacturing. For the environmental microplastics, the leachates may consist of constituent chemicals from each polymer, plastic additives (although likely a different suite from the preconsumer plastics), and any environmental contaminants sorbed from the surrounding environment (e.g., POPs, heavy metals). In each case, the plastic particles were presoaked for 24 h before exposure to the fish, simply by soaking the particles in dechlorinated water at 24 ± 1 °C. For the chemical scenario, the water with the presoaking particles was sieved to remove the plastic particles prior to adding the organisms, such that the organisms were exposed only to the leachates of the plastic. For the physicochemical scenario, the organisms were exposed to both the particles and the leachates. For both exposure scenarios, the test solution (leachates only or particles and leachates) was replaced each day to maintain an equal dose across the 2 scenarios and over the course of the experiment.

Survival and hatching success were recorded daily throughout the experiment. After 14 d, the surviving larvae were euthanized with ethanol. Each replicate group was photographed, dried at 60 °C overnight, and then weighed. The length of each fish was measured using ImageJ (Schneider et al. 2012). The quality of each larva was evaluated based on whether or not it was visibly deformed. The deformities were classified as scoliosis (spinal curvature), edema (fluid buildup around the eyes, heart, and/or yolk sac), failure to hatch, tail truncation, or other (see Supplemental Data for pictures of fish classified as deformed). Each deformity was counted individually such that fish with multiple deformities were counted multiple times. See Figure 1 for examples of each type of deformity.

Statistical analysis

All statistical analyses were done using R, Ver 3.5.1 (R Development Core Team 2018). To ensure that the data fit the assumptions of an analysis of variance (ANOVA), all data were screened for normality (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test). Because the experimental design is asymmetrical (one control group for 2 factors, exposure scenario and dose), individual 2-step ANOVAs were conducted for each endpoint (hatching success, survival, length, weight) and for each experiment (polyethylene, polypropylene, environmental microplastic), using the mean squares from 2 independent ANOVAs, as in Green et al. (2016). First, a one-way ANOVA was performed with all treatments as separate levels ($n = 5$, $N = 25$). Then, a full-factorial 2-way ANOVA was conducted with exposure scenario (2 levels, chemical and physicochemical) and dose (2 levels, low and high) without the control ($n = 5$, $N = 20$). The F ratios were calculated using the residuals from the first ANOVA, which contains the controls. This allows the variation associated with the controls and the other treatments to be distinguished, which is contrasted with 1 degree of freedom (control vs others). Finally, if significance was found for any factor, a Dunnett's test was conducted to compare the control and each level of the significant term. See Supplemental Data, Appendix 1, including Tables S1 to S4 for more information.

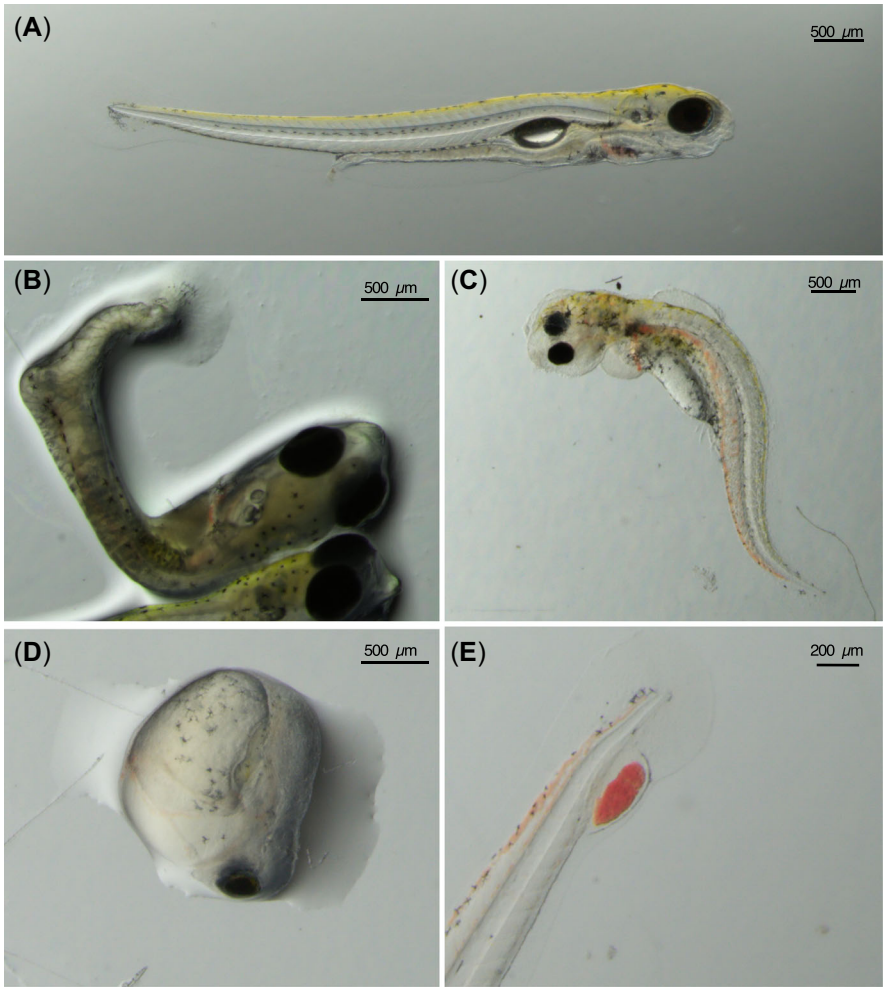

FIGURE 1: Types of deformities compared to a normal larval fathead minnow (**A**). Fish pictured in Figure 1 are exhibiting scoliosis and tail truncation (**B**); edemas around the eyes, yolk sac, and heart (**C**); failure to hatch (**D**); and other (**E**; this was the only deformity classified as "other").

To compare the effects caused by each of the 3 plastics used (polyethylene, polypropylene, environmental microplastic), effect sizes were calculated using R statistical software ("effsize" package; R Development Core Team 2018). Effect sizes compare the mean response of the treatment groups with the mean of the control for each endpoint. Although they are typically used to make results comparable across multiple studies, they are increasingly recognized as a tool to inform the magnitude of the difference between the groups within a study (Sullivan and Feinn 2012). In fact, reporting effect sizes has become standard practice in some disciplines (Appelbaum et al. 2018) and has been recommended as a best practice in all biological journals (Nakagawa and Cuthill 2007). The effect sizes (Hedges' *g*) and 95% confidence intervals were plotted in a forest plot.

RESULTS AND DISCUSSION

We exposed fathead minnows to 3 types of microplastics: preconsumer polyethylene, preconsumer polypropylene, and environmental microplastic, which was a mixture of polyethylene and polypropylene collected from the shoreline of Lake Ontario. Fish were exposed either to the plastic particles and their leachates or to the chemical leachates alone, at a low or a high concentration. The control survival for each test met or exceeded the test acceptability criteria (i.e., 80% survival), supporting the validity of our test conditions. In addition, all data used in statistical analyses passed the tests for normality. The present study shows that 1) the effects of preconsumer microplastics differ by polymer type; 2) the effects of preconsumer and environmental microplastics differ, likely because of the different chemical cocktail associated with each plastic; and 3) the effect of preconsumer microplastics is driven by the physical particle, whereas the effects of environmentally sourced microplastics are caused by both the physical particle and the chemical leachate. By demonstrating that microplastics are both a physical and a chemical stressor, the present study provides support for the growing subset of microplastics research where microplastics are treated as a multiple stressor rather than as a single contaminant (Paul-Pont et al. 2018; Rochman et al. 2019). See Supplemental

Data, Appendix 2, for all data obtained from the 3 experiments.

Effects of preconsumer polyethylene

Larval fathead minnows were significantly affected by exposure to polyethylene microplastic fragments in the high dose of the physicochemical exposure scenario, causing a decrease in survival, length, and weight (Figure 2). Survival in the physchem-high treatment was decreased significantly compared to the other treatments, as indicated by significance in the interaction term ($p = 0.04$). Length was significantly decreased in the high-dose treatments compared to the low-dose treatments, as indicated by significance in the dose term ($p = 0.01$). Weight was significantly decreased in the physicochemical treatments compared to the chemical treatments and in the low-dose treatments compared to the high-dose treatments, as indicated by significance in the exposure scenario term ($p = 0.01$) and the dose term ($p = 0.005$). Compared to the control, the physchem-high treatment had significantly decreased survival (Figure 2A), length (Figure 2D), and weight (Figure 2G), as illustrated by Dunnett's test (Supplemental Data, Table S5). Hatching success was not affected by exposure to preconsumer polyethylene microplastics (Supplemental Data, Figure S1A). Finally, we observed a total of 4 deformities across all fish exposed to preconsumer polyethylene (Table 1; Supplemental Data, Figure S2).

Preconsumer polyethylene microplastics have been shown to cause effects in various aquatic organisms. These include immobilization in *Daphnia magna* (Rehse et al. 2016), reductions in feeding rate and changes to morphology in *Hydra attenuate* (Murphy and Quinn 2018), decreased growth in *Artemia franciscana* (Kokalj et al. 2018), changes to gene expression in zebrafish (although no short-term impacts on larval growth or development were detected [LeMoine et al. 2018]), and increased CYP1A expression in European sea bass (although, again, no impacts on larval growth or development were detected [Mazurais et al. 2015]), among others. In the present study, we saw significant effects from polyethylene exposure only in the physicochemical exposure scenario and only 4 deformities overall. This suggests that the effects of preconsumer polyethylene are driven by an interaction with the particles rather than the chemical compounds associated with the plastic.

Effects of preconsumer polypropylene

Larval fathead minnows were significantly affected by exposure to polypropylene microplastic fragments in the low and high doses of the physicochemical exposure scenario, causing an increase in weight in these treatments (Figure 2H). Weight was increased significantly in the physicochemical-low and physicochemical-high treatments compared with the chemical treatments, as indicated by significance in the exposure scenario term ($p = 0.0002$), and compared to the control, as evidenced by Dunnett's test (Supplemental Data, Table S5). Hatching success (Supplemental Data, Figure S1B), survival (Figure 2B), and length (Figure 2E) were not significantly affected by exposure to polypropylene microplastics. Finally, we observed a total of 6 deformities across all fish exposed to preconsumer polypropylene (Table 1; Supplemental Data, Figure S3).

Preconsumer polypropylene microplastics have been studied to a lesser extent than polyethylene microplastics. Reported effects of polypropylene include increased toxicity in *Hyalella azteca* (fibers [Au et al. 2015]); decreased feeding rate, metabolic rate, and body mass in the Norway lobster (Welden and Cowie 2016); and reduced feeding behavior and energy available for growth in *Carcinus maenas* crabs (fibers [Watts et al. 2015]). The increase in weight in our preconsumer polypropylene experiment is not consistent with previously reported effects for polypropylene microplastics, which have primarily shown negative or neutral impacts. Even expanding to look at all polymer types, it is more common to see a decreased or neutral impact on growth than an increase (Foley et al. 2018), as seen in the present study. One example of a positive impact on growth was reported in *D. magna* exposed to microbeads and fragments of unknown polymer types. These organisms exhibited a decrease in growth in low-food treatments but an increase in growth in high-food treatments. The authors attributed this increase in growth to compensatory feeding in the presence of microplastics (Ogonowski et al. 2016). Shell clams exposed to 6-μm polystyrene microbeads were also shown to experience compensatory feeding, although in this case no increase in condition index was observed (Sussarellu et al. 2016). As such, it is possible that the increase in weight observed in the present study as a result of exposure to preconsumer polypropylene was caused by an increase in feeding due to physical stress of the plastic particles.

Effects of environmental microplastics

Larval fathead minnows were significantly affected by exposure to environmental microplastic fragments in the low dose of the chemical exposure scenario, causing an increase in length and weight, and the high dose of the physicochemical exposure scenario, causing an increase in weight. Length in the chemical-low treatment was increased significantly, as indicated by significance in the interaction term ($p = 0.0084$). Weight was increased significantly in the chemical-low and physicochemical-high treatments, as indicated by significance in the interaction term ($p = 0.0001$). When compared to the control, the chemical-low treatment had significantly increased length (Figure 2F) and weight (Figure 2I), and the physicochemical-high treatment had significantly increased weight, as indicated by Dunnett's test (Supplemental Data, Table S5). Hatching success was not affected by exposure to environmental microplastics (Supplemental Data, Figure S1C). Finally, we observed a total of 35 deformities in fish exposed to environmental microplastic fragments (Supplemental Data, Table S1 and Figure S4).

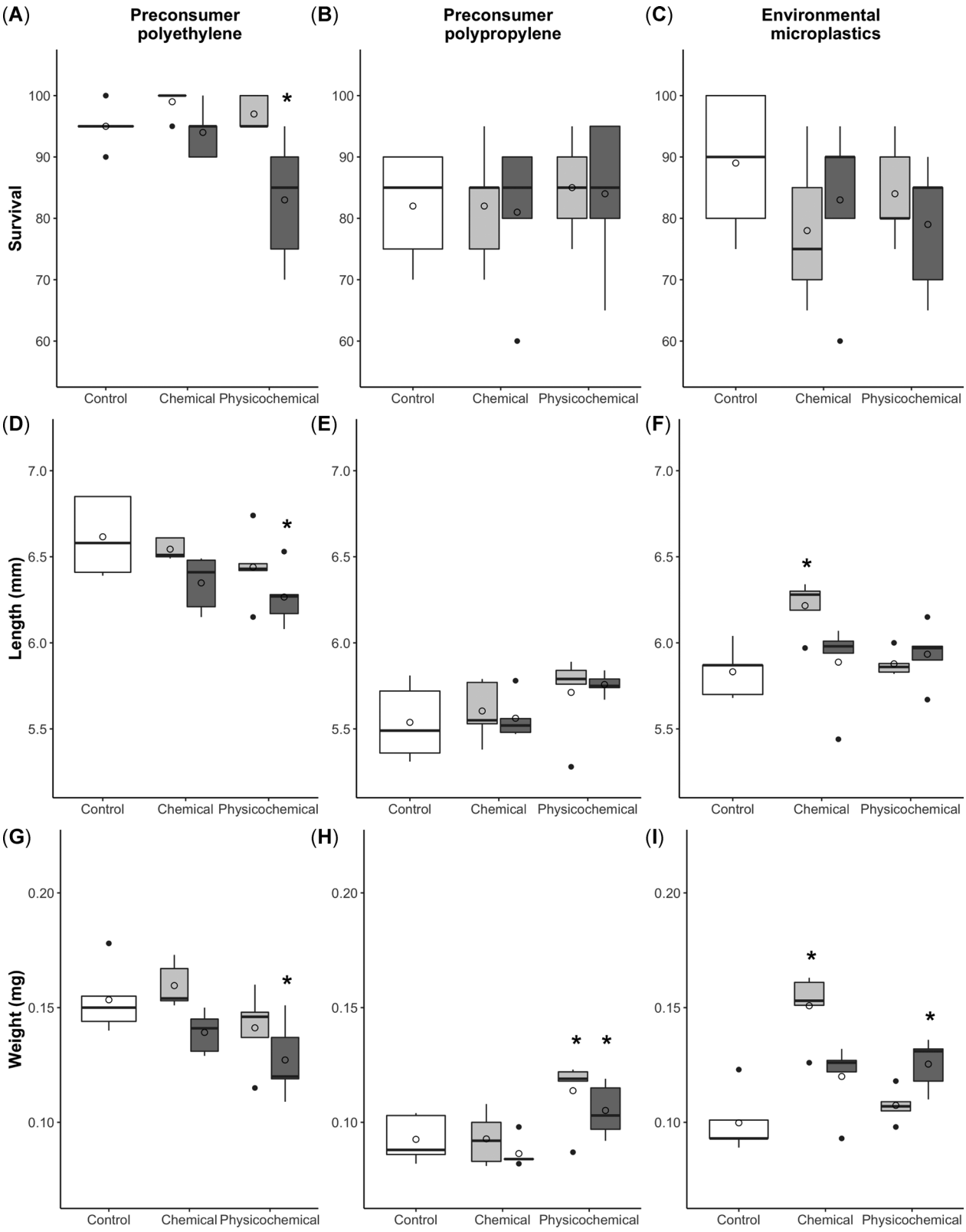

FIGURE 2: Box plots demonstrating the survival (top row), length (middle row), and weight (bottom row) in larval fathead minnows exposed to preconsumer polyethylene microplastics (left column), preconsumer polypropylene microplastics (middle column), or microplastics collected from the shore of Lake Ontario (right column). Low and high doses are represented by the light and dark gray bars, respectively. Open circles represent means, error bars represent standard error, and asterisks denote treatments with a statistically significant difference from the control based on a 2-step analysis of variance ($p < 0.05$).

TABLE 1: Number of deformities in fish exposed to preconsumer polyethylene, preconsumer polypropylene, or environmental microplastics[a]

	Scoliosis	Edema	Hatch	Tail	Other	Total
Preconsumer polyethylene						
Control	0	0	0	0	0	0
Chem-low	0	0	0	0	0	0
Chem-high	0	1	0	0	0	1
PhysChem-low	0	0	0	0	0	0
PhysChem-high	0	3	0	0	0	3
Preconsumer polypropylene						
Control	0	2	0	0	0	2
Chem-low	0	0	0	0	0	0
Chem-high	0	1	0	0	0	1
PhysChem-low	1	1	1	0	0	3
PhysChem-high	0	0	0	0	0	0
Environmental microplastics						
Control	0	1	0	0	0	1
Chem-low	1	4	1	1	0	7
Chem-high	0	5	0	0	1	6
PhysChem-low	1	2	1	2	0	6
PhysChem-high	1	11	2	1	0	15

[a]In each experiment, $n = 5$, with 20 fish per replicate.
Chem-low = chemical exposure, low dose; Chem-high = chemical exposure, high dose; PhysChem-low = physicochemical exposure, low dose; PhysChem-high = physicochemical exposure, high dose.

Although plastic is considered to be biologically inert, plastic in the environment contains a complex mixture of chemicals, including chemicals from the manufacturing process and chemical compounds sorbed from the surrounding environment. These compounds are able to penetrate into cells and react with biologically important molecules in the exposed organism (Teuten et al. 2009). In the present study, we exposed larvae to microplastics collected from the shorelines of Lake Ontario (environmental microplastic). Lake Ontario is the terminal lake in the Laurentian Great Lakes chain, and its watersheds are highly urbanized and industrialized. Consequently, its surface waters and sediments contain legacy contaminants, heavy metals, and newer emerging contaminants. The legacy contaminants found in Lake Ontario include polychlorinated biphenyls (PCBs), perchlorinated dibenzo-*p*-dioxins and dibenzofurans, organochlorine pesticides (e.g., DDT), and heavy metals (e.g., arsenic, mercury, lead; Marvin et al. 2003). Emerging contaminants found in Lake Ontario include pharmaceuticals (Li et al. 2010), flame retardants (Ismail et al. 2009), and perfluoroalkyl compounds (e.g., perfluorinated sulfonate [PFOS], perfluorooctanoic acid [PFOA] [Furdui et al. 2008]). The plastics used in this experiment were collected in Humber Bay, into which flows the Humber River, a major source of pollution to Lake Ontario (Corcoran et al. 2015). Although the plastics used in our experiment were not tested for these compounds, previous studies have shown that polycyclic aromatic hydrocarbons, PCBs, and polybrominated diphenyl ethers can sorb to polypropylene and polyethylene (Mato et al. 2001; Rochman et al. 2013). Additional studies have shown that ingested plastic can act as a vector for environmental contaminants to wildlife, where they can cause biological effects including endocrine disruption (Teuten et al. 2009).

Exposure to low doses of endocrine disruptors can lead to permanent changes to the endocrine system, leading to changes in reproduction and metabolism, and increasing tumor promotion (Gallo et al. 2018). In the present study, we saw increased length, weight, and incidence of deformities in larvae exposed to microplastics assumed to have sorbed environmental contaminants. Although an increase in length and weight is, at first glance, surprising, these results are consistent with the effects of a subset of endocrine disruptors called "metabolism-disrupting chemicals" (MDCs) that control energy homeostasis (Grün and Blumberg 2009; Nadal et al. 2017). These MDCs can disrupt sensitive metabolic processes if exposure occurs during early development (Heindel et al. 2015). Bisphenol-A, for example, was shown to have metabolism-disrupting effects in zebrafish exposed during the embryonic stage, causing an increase in fish length and weight (Riu et al. 2014). Other MDCs that tend to sorb to plastic products in the environment include tributyltin, PCBs, PFOS, PFOA, and phthalates (Nadal et al. 2017), all of which can be found in Lake Ontario (Marvin et al. 2003; Furdui et al. 2008; Ismail et al. 2009; Li et al. 2010). In addition to the chemical cocktail, microplastics in the environment have an associated biofilm that is comprised of microbiota. The biofilm associated with microplastics has been shown to take up chemicals from the environment and act as a barrier for the release of chemicals associated with the particle (Rummel et al. 2017). The biofilm itself may also provide an additional nutritional source to the organism, which could contribute to increased growth. However, the microplastics in the present study were scrubbed and rinsed to remove the biofilm. As such, our findings are more consistent with exposure to metabolism-disrupting compounds associated with microplastic pollution collected from the shoreline of Lake Ontario. Further research is required to understand the mechanisms by which MDCs affect fish and the long-term consequences of MDC exposure.

Comparing across plastic types

Exposure to different types of preconsumer microplastic fragments and microplastics collected from the environment can cause impacts that differ in both type and extent. We show that exposure to preconsumer polyethylene caused a decrease in survival (effect size −1.40), length (−1.58), and weight (−1.49) in the physicochemical-high treatment (Figure 3). Exposure to preconsumer polypropylene caused an increase in weight in the physicochemical-low (1.48) and -high (1.04) treatments. In the environmental microplastic treatment, larvae experienced a significant increase in length (2.36) and weight (3.24) in the chemical-low treatment and an increase in weight in the physicochemical-high treatment (1.86). Finally, we saw 5.8 times as many deformities in the environmental plastic treatment compared to the preconsumer treatments.

Few studies have explicitly compared the effects of different polymer types while holding all other variables (size, shape, concentration in particles per volume) constant. Polymer type has been suggested as an important factor driving the impacts of microplastics on the basis of how chemically harmful the monomers that make up the polymer are (Lithner et al. 2011).

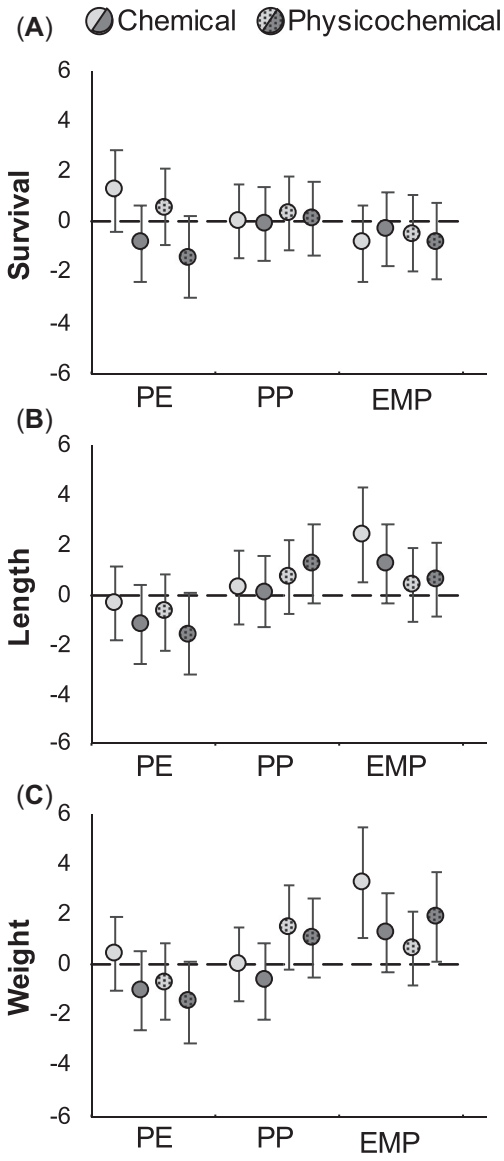

FIGURE 3: Forest plots demonstrating the differing impacts of the low dose (light gray) and high dose (dark gray) of polyethylene, polypropylene, and environmental microplastics on larval survival (**A**), length (**B**), and weight (**C**). Effect sizes were calculated as Hedges' *g*, and error bars represent 95% confidence intervals. PE = polyethylene; PP = polypropylene; EMP = environmental microplastics.

Although polyethylene and polypropylene are considered to be among the least hazardous polymer types (Lithner et al. 2011), they can nonetheless cause physical impacts, as seen in the present study. One previous study compared the differing effects of preconsumer polyethylene and polypropylene to microalgae (Lagarde et al. 2016). In their study, they found that polypropylene microplastics caused a slight but significant decrease in growth and an overexpression of genes involved in sugar biosynthesis, an effect that was seen to a larger extent in the polyethylene treatment. Furthermore, they demonstrated more cohesive colonization of microalgae on polypropylene compared to polyethylene, which the authors attributed to the differences in gene expression (Lagarde et al. 2016). Although the authors note that it was unclear why they observed differences between polymer types, they suggest that the exacerbated impacts in the polypropylene treatment could be due to increased particle aggregation (Lagarde et al. 2016)—in other words, a physical, rather than chemical, effect. In our experiment, it is also unclear why we saw differing, and even opposite, impacts in the preconsumer polyethylene and polypropylene experiments. Because the effects were only seen in the physicochemical exposure scenarios, it is possible that the difference in effects may be due to morphological differences between the 2 plastic types. In our experiment, the polyethylene fragments resembled more fibers and films, whereas the polypropylene fragments had a more typical fragment shape and a rubbery feel (Figure 4). Because fibers have been demonstrated to be more harmful than fragments (Gray and Weinstein 2017), it is possible that the decrease in length and weight in the polyethylene experiment was due to the harmful impacts of the polyethylene fragment shape. Difference in particle morphology may have acted in tandem with compensatory feeding, the mechanism we suggest caused an increase in weight in the polypropylene experiment. Thus, polyethylene microplastics may have been more physically harmful than polypropylene microplastics and caused more severe impacts that could not be overcome by compensatory feeding, resulting in decreases in survival, length, and weight. On the other hand, polypropylene may have been slightly less physically harmful and thus had no effect on survival, and compensatory feeding resulted in an increase in larval weight.

Although the vast majority of microplastic studies have investigated the effects of preproduction microplastics, a few

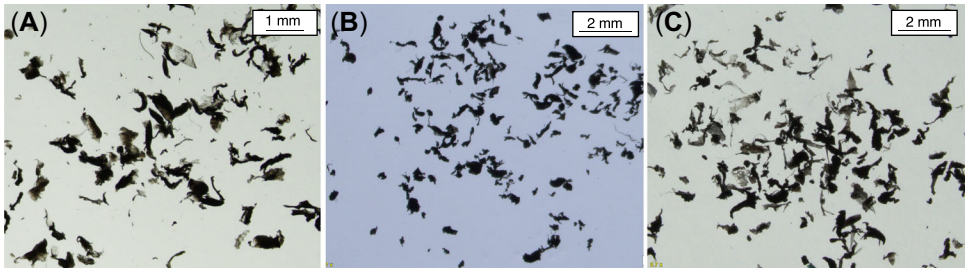

FIGURE 4: Polyethylene (**A**), polypropylene (**B**), and environmental microplastics (**C**).

studies have compared the effects of preproduction and environmental microplastics. Not only are these microplastics more environmentally realistic, but they are also reported to be consumed more readily than preconsumer microplastics (Vroom et al. 2017) and internalized into cells more frequently (Ramsperger et al. 2020). They have also been suggested to affect organisms more severely. For example, in Japanese medaka exposed to both preproduction and environmental polyethylene fragments, the fish in both treatment groups showed signs of stress in the liver but with greater effects observed in fish exposed to environmental microplastics (Rochman et al. 2013). Another study investigated intestinal damage in European sea bass exposed to preconsumer and environmental polyvinyl chloride (PVC) particles and found increased effects in fish exposed to the environmental microplastics (Peda et al. 2016). In these studies, environmental microplastics were seen to exacerbate the severity of the effects of the preconsumer microplastics. Similarly, we found that the environmental microplastics (a mixture of polyethylene and polypropylene) caused an exacerbated impact compared to polypropylene and a more severe number of deformities, but the opposite effect compared to polyethylene.

The impacts of microplastics can be driven by physical and/or chemical processes, depending on the formulation of the plastic. In the present study, we show that effects of preconsumer microplastics were driven by the physical impacts of the plastic. In the environmental microplastics experiment, where plastic was collected from the shoreline of Lake Ontario, impacts were driven by the chemical leachates as well as potentially the physical particles (as seen in the physicochemical treatments, where fish were exposed to both the physical particles and their leachates). As discussed previously, some studies have compared the effects of microplastics with and without sorbed environmental contaminants. However, few studies have exposed organisms to only the cocktail of contaminants associated with preconsumer and/or environmental microplastics. One study compared the acute toxicity of leachates of newly purchased plastic products consisting of one of 5 plastic types. They found the leachates associated with PVC products to be highly toxic to *D. magna* but the leachates associated with polypropylene products and 4 out of 5 polyethylene products to be nontoxic (Lithner et al. 2012). These results suggest that the leachates alone of some plastic products can be hazardous to wildlife, but the leachates of polyethylene and polypropylene microplastics are relatively harmless until the products are exposed to the environment where they can sorb persistent, bioaccumulative, and toxic substances.

As a result of the design of our study, it was difficult to predict the effects of the environmental plastics because preconsumer polyethylene and polypropylene alone produced opposite effects. If the effects seen in the environmental microplastic experiment were simply due to the polyethylene and polypropylene polymers and if the effects of polyethylene and polypropylene were additive, we might have expected to see a neutral outcome because each plastic type alone caused effects opposite to the other. Instead, we see an overall positive impact from the environmental microplastic that is larger in magnitude than the changes seen for preconsumer polypropylene and opposite to polyethylene. We also see roughly 6 times the number of deformities than either preconsumer treatment. These results might be due to a synergistic effect caused by the combination of polyethylene and polypropylene polymer types and/or to the chemicals associated with the plastic in the environmental microplastic experiment. The latter of these 2 explanations is further evidenced by the significant effects seen in the chemical exposure scenario of the environmental microplastic treatment and not in the chemical exposure scenarios of either of the preconsumer treatments. Further research is required to distinguish between these 2 possible explanations. Still, the results of these experiments indicate that there is a difference in the chemical leachates between the preconsumer and environmental microplastics that is driving the increase in length, weight, and deformities. Because we do not know the mixture of chemicals on the particles or leachates, we cannot say specifically which chemicals are responsible for the observed effects. However, if we had done chemical analysis, we would only know a subset of the chemicals associated with the plastic based on the methods of our analysis (whether targeted or untargeted), and it is nearly impossible to unveil the full chemical cocktail associated with the environmental microplastics. Finally, if we had soaked the plastics for longer than 24 h or dosed at higher concentrations, we may have seen stronger effects. Still, our results show that environmental microplastics are more harmful than preconsumer, or "virgin," microplastics, suggesting that the added toxicity is related to the complex mixture of contaminants from the environment.

Microplastics are a multiple stressor

The present study demonstrates that the impacts of microplastics are dependent on both polymer type and the presence of sorbed environmental contaminants and that the impacts can be both physical and chemical in nature. As such, we contend that studies trying to better understand the effects of microplastics on wildlife should treat microplastics as a multiple stressor with both physical and chemical stressors rather than a single contaminant. Microplastics exist in a near-infinite number of formulations, which need to be carefully considered when designing an experiment to investigate effects. The characteristics of microplastics that will drive whether or not they cause an effect include the size distribution of particles (smaller particles are more harmful than larger particles [Earn et al. 2020]), shape (fibers are more harmful than fragments, which are more harmful than spheres [Gray and Weinstein 2017]), polymer type (e.g., PVC is more harmful than polyethylene and polypropylene [Lithner et al. 2011]), additives (newly purchased plastic products may be more harmful than preproduction pellets because of additives such as flame retardants and plasticizers in the finished product [Lithner et al. 2011]), and environmental contaminants (environmentally sourced microplastics are more harmful than preconsumer

pellets [Rochman et al. 2014]). The term "microplastics" refers to a suite of environmental contaminants with different sources, characteristics, and environmental and biological fates. As such, generalizing about the effects of "microplastics" should be avoided, and the results of an experiment should be communicated with precision and specificity.

Future directions

The present study adds to the growing body of literature investigating the effects of microplastics in fish, which have reported negative, neutral, and positive impacts with different plastic types (primarily polyethylene and polystyrene) and with and without sorbed environmental contaminants (Foley et al. 2018; Bucci et al. 2020; Jacob et al. 2020). Following the present study, future work should investigate the effects of preconsumer and environmental microplastics in a chronic exposure to see how the effects seen in the present study unfold over the fish's entire life cycle, including reproduction. In general, more research is required to further investigate the effects of microplastics in fish, which are exposed to microplastics not only via direct ingestion but also through trophic transfer (Gouin 2020). This research should be done strategically, such that we can parse out the effects of different types, shapes, and sizes of microplastics. Effects testing should be done using environmentally realistic types, and concentrations of particles should be reported in particles per liter to facilitate comparison across effects studies and with concentrations in nature. Finally, environmental realism can be further improved by testing microplastics with sorbed environmental contaminants, which can be obtained by aging preconsumer pellets in a body of water (as in Rochman et al. 2013) or by collecting pieces of plastic from the environment, as in the present study, and grinding them into microplastics. An additional treatment for these studies could include exposure to the environmental water alone, to further understand the role of microplastics as a vector for environmental contaminants. It should be noted, however, that neither of these methods perfectly replicates the complexity of microplastics in the environment. However, this is the nature of laboratory experiments, and it remains important to strive toward environmental realism where possible.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5036.

Acknowledgment—We acknowledge financial support from the Ontario Ministry of the Environment, Conservation and Parks (grant COA 2310). We thank D. Green and T. Underwood for guidance on statistical design. We also thank D. Poirier, P. Helm, R. Chong-Kit, and the rest of the Aquatic Toxicology Unit staff for their assistance and support during the experiments and for tolerating the sound of grinding plastic every morning.

Author Contributions Statement—K. Bucci and C. Rochman conceived of and designed experiments and performed statistical analyses; K. Bucci and J. Bikker performed experiments; K. Stevack and T. Watson-Leung provided in-laboratory assistance during experiments and maintained fish cultures from which experimental fish were taken; C. Rochman supervised and obtained financial support; K. Bucci wrote the manuscript. All authors provided editorial assistance for the manuscript.

Data Availability Statement—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.5036.

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